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Genetic change in the open reading frame of bovine viral diarrhea virus is introduced more rapidly during the establishment of a single persistent infection than from multiple acute infections☆

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ABSTRACT

Bovine viral diarrhea viruses (BVDV) are ubiquitous viral pathogens of cattle with a high degree of sequence diversity amongst strains circulating in livestock herds. The driving force behind change in sequence is not well established but the inaccurate replication of the genomic RNA by a viral RNA polymerase without proof-reading capabilities as well as immune pressure on immunodominant proteins are thought to play major roles. Additionally, it is not clear when the majority of changes are introduced, whether during acute infections with exposure to innate and adaptive immune responses or in establishment of persistent infections (PI) in utero. To examine which generates greater sequence diversity, two groups of viruses were compared. The first was six isolates of a single strain of BVDV-2 that were isolated over greater than a year's time. These viruses caused a series of severe acute (SA) BVD outbreaks over a large geographic area. Changes in nucleotide sequence were determined by comparison of the sequence of each strain to the six virus consensus sequence. The second group was composed of six BVDV strains isolated from PI calves whose dams were exposed to PI cattle. Changes were identified by comparison of the sequence of the progenitor PI virus to that of the progeny viruses from the single in vivo 'passage'. The open reading frames (ORF) of the six SA isolates were >99% identical at the nucleotide level with 30% of the changes being nonsynonymous changes. The amount of genetic change increased with time and distance from the original outbreak. Similarly, the PI viruses isolated from single passage PI calves had >99% identity with the progenitor virus. The number of nucleotide changes in these viruses was equal to or greater than that observed in the SA viruses. The majority of the nonsynonymous changes were found in the structural proteins, with 65% of these occurring in the immunodominant E2 protein. Antigenic mapping studies using a monoclonal antibody panel specific for the BVDV E2 protein showed no antigenic differences amongst the six SA viruses, nor between the progenitor and progeny type 1a and type 2 persistent viruses. However, antigenic differences were observed in the two type 1b progeny viruses that possessed the greatest number of amino acid changes. Two antibodies were found to have altered staining patterns. These results suggest that the establishment of a single persistent infection results in more rapid generation of genetic diversity in BVDV strains than a series of acute infections and may contribute to antigenic change in the absence of an immune response.

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1. Introduction

Bovine viral diarrhea virus (BVDV), a member of the Pestivirus genus of the *Flaviviridae*, has a worldwide distribution, infecting a large number of ruminant as well as non-ruminant species. BVDV are currently classified into two genotypes, 1 and 2, where genotype 1 is further divided into more than 11 subgenotypes while genotype 2 is divided into 2a and 2b (Flores et al., 2002; Vilcek et al., 2001, 2005). Additionally, BVDV exists as two biotypes, cytopathic and noncytopathic, based on their ability to cause cell death in cultured cells.

All BVDV contain a single-stranded, plus sense, non-polyadenylated RNA genome of greater than 12 kb in size. The genomic RNA encodes a single large polyprotein that is co- and post-translationally cleaved to produce the mature viral proteins. The viral proteins are encoded in the genomic RNA in the order NH_2-N^{pro}-capsid-E^{rns}-E1-E2-p7-NS2/3-NS4a-NS4b-NS5a-NS5b-

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COOH (Collett et al., 1988). The capsid, E^{rns}, E1 and E2 proteins are structural proteins while the remainder are nonstructural and are involved in viral replication and host defense evasion.

One important aspect of BVDV is the ability of noncytopathic strains of either genotype to establish immunotolerance in first trimester fetuses resulting in lifelong persistent infections. PI cattle are considered the primary means of spreading BVDV in domestic livestock herds. Exposure of a pregnant dam to BVDV results in an acute, systemic infection that crosses the placenta infecting the fetus. For persistence to be established, infection must take place before maturation of the fetal immune system, infections taking place later are cleared and the calf is born with immunity to BVDV (Kahrs et al., 1970; McClurkin et al., 1984). Interestingly, PI animals are able to respond to infection by BVDV strains of other subgenotypes but may not show a response to viruses of the same subgenotype as the persistent virus (Fulton et al., 2003).

The disease caused by BVDV ranges from clinically inapparent to severe acute (SA) disease. A series of SA BVD outbreaks caused by noncytopathic BVDV type 2 viruses occurred in Quebec, Canada in the early 1990s that caused great economic losses in veal calf operations with losses occurring in adult animals as well (Pellerin et al., 1994). In 1993, the mortality rate in veal calves increased significantly where 32,000 out of 143,000 calves died. Type 2 BVDV was also associated with SA disease in a large outbreak in Ontario, Canada that caused deaths in all age groups of cattle. It was estimated that 150 dairy, 600 beef and 100 veal operations were affected (Carman et al., 1998). Outbreaks of type 2 SA disease with high mortality similar to that observed in Canada also occurred in the US later in 1993 beginning in New York state following importation of a dairy heifer from Canada. SA BVD outbreaks then spread west to Indiana and Iowa (Odeon et al., 1999; Ridpath et al., 2006). In a study examining the genomic RNAs of BVDV type 2 strains causing SA disease, three viruses from the Ontario, Canada and New York outbreaks (1373, 24515, NY-93) were found to possess the same unusual 60 base insert of unknown origin within the NS2/3 coding sequences (Ridpath et al., 2006). Further comparison revealed that the remainder of the genomic RNAs from these three viruses shared greater than 99% sequence identity. PCR analysis was used to examine additional type 2 SA isolates for the presence of this small insert in the NS2/3 region and three additional viruses were identified. These viruses, IAF-103, 793, and 37621, were isolated in Quebec, Canada from the original outbreak, Indiana and Iowa, respectively, and possessed greater than 99% identity with the original three viruses. This revealed that rather than being unique strains of SA BVDV type 2 causing individual outbreaks at different locations, these viruses were the same strain that spread through a large geographic area over greater than a year's time (Ridpath et al., 2006). This was the first study to follow the same strain through a number of different outbreaks.

Multiple studies have been conducted that looked at generation of sequence diversity in BVDV; however, most have been limited to viruses isolated from persistently infected animals and to small regions of the 5′ untranslated region and the E2 protein (Collins et al., 1999; Hamers et al., 1998; Jones et al., 2002; Paton et al., 1995). Within PIs, little change is observed or believed to take place because immune surveillance forces maintenance of the original viral sequence and tolerates little change (Paton et al., 1994). Other studies revealed that there were small numbers of nucleotide changes with some resulting in amino acid substitutions from the strain infecting the dam but the virus then remained stable over time (Paton et al., 1994). In studies where the genetic diversity of BVDV causing acute disease were examined, the focus was on strains circulating in livestock herds that were unrelated genetically and antigenically (Hamers et al., 1998). These studies found significant variation at the genetic and antigenic levels between herds with a higher degree of relatedness within herds. Acute infections were believed to be the major source of genetic variation in BVDV (Bolin et al., 1991; Bolin and Ridpath, 1992).

Here, the complete ORF of six SA isolates and nine viruses from persistently infected calves were sequenced to gain insight into the rate of nucleotide substitutions and amino acid changes between acute and persistent infections. The rate of sequence change in viruses isolated from SA disease outbreaks was compared to that of viruses isolated from a single 'passage' in persistently infected calves. Characterization of antigenic differences between viruses was done using a 27 monoclonal antibody panel reactive to the E2 protein.

2. Materials and methods

2.1. Cells and virus

BVDV strains were isolated from persistently infected calves on Madin Darby bovine kidney cells (MDBK) and were genotyped as previously described (Rodning et al., 2010). Growth in cell culture was limited to 1 passage to limit possible nucleotide changes in the genomes of the viruses.

The severe acute (SA) type 2 BVDV strains used here were IAF-103, 1373, 24515, NY-93, 793 and 37621. Severe acute (SA) BVDV strains were isolated on MDBK cells as previously described (Ridpath et al., 2006).

2.2. Reverse transcriptase PCR and DNA sequencing

BVDV genomic RNA was isolated from viral stocks using the Qiagen viral RNA isolation kit (Qiagen, Inc., Valencia, CA) as described by the manufacturer. The RNA was used as template in reverse transcriptase PCR (rtPCR) reactions to amplify specific regions of the genome for sequence analysis. The 50 μl rtPCR reactions consisted of 1X Pfx50 buffer, 2.5 mM dNTPs, forward and reverse primer (2 pM), 4 μl of purified viral RNA, 40 units of RNase inhibitor (Promega Inc., Madison, WI), 200 units of Superscript III (Invitrogen, Inc., Carlsbad, CA) and 5 units of Pfx50 DNA polymerase (Invitrogen). The rtPCR reactions were run by first heating to 56 °C for 20 min, then 94 °C for 1 min, followed by 40 cycles of 94 °C for 10 s, 55 °C for 15 s and 70 °C for 1 min. After completion of cycling, the PCR reactions were run on 1% agarose/ethidium bromide gels and the amplicons were excised and purified using a GeneClean spin column kit (MP Biomedicals, Inc., Solon, OH). DNA sequencing was done using an ABI PRISM 3100 automated sequencer with ABI BigDye Terminator v3.1 chemistry (ABI, Inc., Carlsbad, CA). The amplicons were produced and sequenced twice. The sequences were edited and assembled using CodonCode Aligner software (Codoncode, Inc., Dedham, MA). The origin of all viruses and GenBank accession numbers are given in Table 1.

2.3. Immunocytochemistry

Characterization of the antigenic properties of the SA and persistent viruses was done using a panel of monoclonal antibodies that were reactive to the BVDV E2 protein and was done as previously described (Ridpath et al., 1994).

3. Results

3.1. Viruses from persistently infected calves

The open reading frame (ORF) encoded by the genomic RNAs of three persistent progenitor viruses and 6 progeny viruses were sequenced in this study. The 3 progenitor viruses were 180 (type 1a), 526 (type 1b) and 446 (type 2). The calves persistently infected

Table 1
PI-derived and severe acute BVDV strains.

Virus	Origin	GenBank accession #
PI progenitor virus		
180	Alabama	HQ174292
446	Alabama	HQ174297
526	Alabama	HQ174294
PI progeny virus		
8824	Alabama	HQ174295
8827	Alabama	HQ174298
8830	Alabama	HQ174296
8831	Alabama	HQ174299
8833	Alabama	HQ174300
8844	Alabama	HQ174293
Severe acute virus		
IAF103	Quebec	HQ174301
1373	Ontario	AF145967
24515	Ontario	AY149216
NY-93	New York	AF502399
793	Indiana	HQ174302
37621	Iowa	HQ174303

with these viruses were commingled with pregnant, BVDV naïve heifers between 68 and 126 days after artificial insemination (Rodning et al., 2010). Viruses were isolated from six of the resulting 12 persistently infected calves and were used here. Preliminary typing showed that there was a single type 1a progeny virus (8844), two type 1b progeny viruses (8824 and 8830) and three type 2 progeny viruses (8827, 8831 and 8833).

3.2. Sequence analysis of progenitor and progeny persistent viruses

The nucleotide sequence of the open reading frame (ORF) was determined for the nine persistent viruses. All viruses within species or subspecies were highly similar to each other (>99%) with a varying number of nucleotide substitutions (Table 2). The sequences of progenitor and progeny viruses were compared to determine the extent of nucleotide substitutions that occurred in establishing the persistent infection. The number of nucleotide substitutions ranged from 17 to 48 per ORF (0.15–0.4%). These changes were, for the most part, evenly distributed throughout the ORF. The majority of the changes were found in the wobble position of the codon, resulting in no change in encoded amino acid. The positions of the nucleotide substitutions in the ORF that resulted in amino acid changes as well as the corresponding amino acid changes for each PI virus are listed in Table 2. Nucleotide substitutions resulting in amino acid changes were slightly more prevalent in the portion of the ORF encoding the structural proteins with progeny strain 8831 being the exception. Additionally, 38 of 69 amino acid changes occurred in the structural proteins with 25 of these changes occurring in the E2 protein. The majority of nucleotide and amino acid changes were unique to the individual viruses. However, there were substitutions that were common amongst viruses. In viruses 8824 and 8830, nucleotide 2714C of progeny strain 526 was replaced with U resulting in the change S905F. Base position 2978 G was replaced with A in strain 8824 and 2979 A was placed with C in strain 8830 resulting in R993K and R993S, respectively. The same amino acid residue 993R was changed to lysine and serine by two different bases changes. In the type 2 strains, all three progeny viruses possessed the 2647 A to G substitution that resulted in the N883D amino acid change. All of these common amino acid changes were found in the E2 protein.

3.3. Sequence analysis of severe acute BVDV strains

The complete nucleotide sequence of SA BVDV strains 1373, NY-93 and 24515 were obtained from GenBank (Table 1). The nucleotide sequence of the open reading frame of SA BVDV strains IAF-103, 793 and 37621 were determined here. Identification of changes in each viral genome was made by comparison of each sequence to the six virus consensus sequence in order to compare all viruses to the same sequence. As noted with the BVDV strains isolated from PI animals, there was a limited degree of nucleotide sequence divergence amongst these viruses ranging from 4 to 33 nucleotides (0.04–0.3%). Most nucleotide changes in these viruses resulted in no amino acid substitutions. The nucleotide changes that resulted in amino acid substitutions were found primarily in the nonstructural proteins (Table 3). Only 6 of the predicted 34 amino acid changes occurred in the E2 immunodominant protein.

Based on the amino acid sequences of the SA strains, it was possible to derive a rough genealogy for these viruses. IAF-103, the first SA BVDV strain isolated from the 1993 outbreak in Quebec, Canada (exact date unknown) was the closest to the consensus sequence with only 4 nucleotide differences and 1 amino acid substitution (Table 3). The next closely related viruses were 1373 and 24515, both of which were isolated in Ontario, Canada during the same period of time. These two viruses had 14 and 16 nucleotide substitutions, respectively with 6 amino acid changes each. Also, 6 nucleotide and 3 amino acid changes were in common. These were the only two viruses that had identical changes, indicating they diverged in Ontario at roughly the same time. The viruses isolated in the US were more divergent, indicating a longer period of time or a larger number of acute infections. NY-93 had the greatest number of nucleotide and amino acid changes and may have been isolated some time after its introduction to New York. The other two US strains did not possess as many changes as NY-93 but were more divergent from the consensus sequence then the Canadian isolates. The greater number of changes noted in the US-isolated strains indicated that a greater amount of time and number of acute infections occurred as the virus was spread south and then west of the original point of isolation.

3.4. Comparison of genomic changes in severe acute and persistent viruses

The comparison of the number of nucleotide and amino acid changes that occurred in SA viruses that passed through a large geographical area and an unknown number of acute infections with viruses that were passed a single time in PI animals was very revealing. A single viral ‘passage’ in the production of a PI animal resulted in at least equivalent numbers of nucleotides changes as compared to the SA viruses. In fact, two PI viruses had more nucleotide changes and five had more amino acid changes then that observed amongst the entire group of SA viruses. Forty-one percent of nucleotide changes in the PI viruses resulted in amino acid changes as compared to 30% in SA viruses. Most of the amino acid changes in the PI viruses occurred in the structural proteins with most of these in the E2 protein (37% of total). Most amino acid changes in the SA viruses occurred in the nonstructural proteins with only 17% of amino acid changes found in the E2 protein. This illustrates the difference between PI derived and SA viruses in the proteins that had the greater numbers of amino acid changes.

3.5. Characterization of antigenic relationships with E2 monoclonal antibodies

A panel of 27 monoclonal antibodies reactive to the E2 protein of BVDV was used to examine antigenic characteristics of the viruses used in this study. There were no changes in staining patterns of any of the monoclonal antibodies with the SA viruses. There were no more than two amino acid changes from the six virus consensus sequence in any one virus making the probability of altering antibody recognition small.

Table 2
Positions of nucleotide and amino acid changes in progeny viruses.

Progenitor:Progeny	Nucleotide differences	Nucleotide position and change[a]	Amino acid position and change[b]	Viral protein
180[c]:8844	27 (9 aa changes)	1180 G:A, 1741 G:A, 1898 C:T, 2863 A:G, 3061 G:A, 6178 T:A, 6403 G:A 9830 T:A, 10093 A:G	394 V:I, 581 V:I, 633 A:V 955 I:V, 1021 D:N, 2060 L:M 2135 I:V, 3277 I:K, 3365 N:D	E^{rns}, E1, E1 E2, E2, NS3 NS3, NS5b, NS5b
526[d]:8824	48 (19 aa changes)	988 G:T, 1203 T:C, 1315 C:G, 1330 G:T, 1660 G:A, 2680 G:A, 2714 C:T, 2912 A:G, 2917 T:C, 2978 G:A, 3194 C:G, 3487 A:G, 4262 A:G, 4456 A:G, 4645 A:G, 6820 T:C, 9851 G:A, 9925 C:T, 10681 G:A	330 A:S, 401 S:R, 439 H:D, 444 V:L, 554 V:M, 894 I:V, 905 S:F[e], 971 K:R, 973 Y:H, 993 R:K[f], 1065 S:G, 1163 I:V, 1420 T:A, 1486 I:V, 1549 T:A, 2274 S:T, 3284 R:K, 3309 H:Y, 3609 D:N	E^{rns}, E^{rns}, E^{rns} E^{rns}, E1, E2, E2, E2, E2, E2, NS2 NS3, NS3, NS3, NS3, NS5b, NS5b, NS5b
526:8830	35 (16 aa changes)	341 T:C, 680 T:C, 1675 G:T, 2017 G:T, 2096 G:A, 2225 A:G, 2246 C:T, 2338 G:A, 2714 C:T, 2979 A:C, 3418 G:A, 3662 T:C, 5437 A:G, 7120 G:A, 7153 A:G 10102 T:G	114 I:T, 227 V:A, 559 V:L, 673 V:L, 699 G:D, 742 D:G 749 T:I, 780 E:K, 905 S:F,[e] 993 R:S[f], 1140 A:T, 1221 V:A 1813 I:V, 2374 E:K, 2385 K:E, 3368 L:V	N^{pro}, capsid, E1, E2, E2, E2, E2, E2, E2 E2, NS2, NS2, NS3, NS4b, NS4b, NS5b
446[g]:8827	17 (9 aa changes)	1118 G:A, 1952 G:A, 2605 T:C, 2647 A:G, 2825 T:C, 3008 G:A 8365 G:A, 8932 A:G, 10651 A:T	373 G:E, 651 R:K, 869 Y:H, 883 N:D[h], 942 I:T, 1003 R:Q, 2789 I:V, 2978 V:M, 3551 I:L	E^{rns}, E2, E2, E2, E2, E2, NS5a, NS5a, NS5b
446:8831	17 (10 aa changes)	220 G:A, 2170 T:C, 2330 C:T, 2647 A:G, 3245 G:A, 4087 G:A, 4381 A:G, 5608 A:T, 6772 G:A, 7477 C:A	73 V:M, 724 L:P, 777 T:I, 883 N:D[h], 1082 S:N, 1363 V:M, 1461 I:V, 1870 M:L, 2258 V:I, 2493 L:M	N^{pro}, E2, E2, E2, NS2, NS2, NS2, NS3, NS4a, NS4b
446:8833	22 (6 aa changes)	439 G:A, 1451 A:G, 1741 A:G, 2647 A:G, 2672 T:C, 7359 T:G	147 D:N, 484 K:R, 581 I:V, 883 N:D[h], 891 L:P, 2453 I:M	N^{pro}, capsid, E1, E2, E2, NS4b

[a] Position of nucleotide change in open reading frame (ORF).
[b] Position of amino acid change in ORF.
[c] 180/8844: type 1a viruses.
[d] 526/8824/8830: type 1b viruses.
[e] Amino acid residue #905 changed from serine to phenylalanine in both 8824 and 8830.
[f] Amino acid residue #993 changed in 8824 from arginine to lysine and in 8830 from arginine to serine.
[g] 446/8827/8831/8833: type 2 viruses.
[h] Amino acid residue #883 changed from asparagine to glutamic acid in 8827, 8831 and 8833.

Table 3
Positions of nucleotide and amino acid changes in severe acute viruses.

Virus	Nucleotide differences[a]	Nucleotide position and change	Amino acid position and change	Viral protein
IAF-103	4 (1 aa change)	3457 A:G	1153 T:V	NS2
24515	16 (6 aa changes)	2602 A:G[b], 4352 C:T, 4405 A:G[b], 8118 A:G,10602 G:A[b], 10712 A:C	868 I:V[c], 1451 A:L, 1469 T:A[c], 2706 I:V, 3534 V:I[c], 3571 T:P	E2, NS2, NS2 NS5a, NS5b, NS5b
1373	13 (6 aa changes)	2126 A:G, 2602 A:G[b], 4405 A:G[b] 5180 A:T 9465 G:A, 10602 G:A[b]	709 K:R, 868 I:V[c], 1469 T:A[c], 1727 D:N, 3155 D:N, 3534 V:I[c]	E2, E2, NS2 NS3, NS5a, NS5b
793	19 (5 aa changes)	7357 T:C, 8755 T:C, 8785 T:A 9522 G:T	2452 V:A, 2918 G:W, 2928 V:E, 23174 G:W	NS4b, NS5a, NS5a, NS5b
NY-93	33 (7 aa changes)	1580 A:G, 2698 A:G, 3305 T:C, 4313 T:C, 6024 T:C, 7459 C:T 10702 C:T	527 K:R, 900 Y:H, 1102 V:A, 1438 V:A, 2008 V:A, 2486 R:K 3567 T:I	E1, E2, NS2, NS2, NS3, NS4b, NS5b
37621	27 (9 aa changes)	1936 A:G, 2605 T:C, 2907 C:A, 3763 C:T, 4436 A:C, 4604 G:A, 4661 G:A 9463 A:G, 9735 G:A	656 M:V, 869 Y:H, 969 S:R, 1255 L:F, 1479 N:D, 1535 V:M[b], 1554 K:E, 3154 N:R, 3245 G:R	E1, E2, E2, NS2, NS2, NS3 NS3, NS5a, NS5b

[a] Difference compared to 6 virus consensus sequence.
[b] Nucleotide changes in common between strains 1373 and 24515.
[c] Amino acid changes in common between strains 1373 and 24515.

There was no change in recognition of the monoclonal antibodies between progenitor and progeny type 1a and type 2 viruses. However, there were two antibodies of the panel that showed altered staining with two progeny type 1b viruses. Progeny virus 8824 gained recognition with antibody CA-80 while PI virus 8830 lost staining with antibody CA-36. These type 1b viruses possessed a greater number of amino acid changes in the E2 protein then did the SA viruses or the other progeny viruses. These changes apparently introduced antigenic change in the E2 protein in the absence of an immune response. This monoclonal antibody analysis was not exhaustive; there exists the possibility that antigenic changes occurred in additional epitopes that were not detectible by the antibodies available for this study.

4. Discussion

It is well established that BVDV strains mutate and change rapidly; however, it is not clear what drives this rapid change. Most often changes are attributed to a sloppy, non-proof reading RNA dependent RNA polymerase. Multiple cycles of replication lead to a population of viruses with minor differences, termed the quasispecies (Domingo et al., 1985). With a large population of virus genomes, each possessing slightly different nucleotide and amino acid sequences, the virus has the ability to adapt rapidly to new circumstances by having a genomic sequence available that can survive or adapt to the new assault (Collins et al., 1999; Domingo, 1998; Domingo et al., 1998). This provides a relative advantage and the selected sequence rapidly becomes predominant. However, it

is unknown if a sloppy polymerase is the only mechanism acting to produce altered sequences or if other, undiscovered or uncharacterized means exist that influence the mutational frequencies within BVDV genomic sequences.

In this report, the ORF of six type 2 BVDV isolated from a series of severe acute outbreaks were compared to determine the rate of change with increasing geographic distance and time from the original outbreak. This is the first study to compare complete ORF sequences of BVDV isolates comprising a single strain from a widespread series of acute outbreaks to examine sequence divergence. This, to our knowledge, is the only series of outbreaks that can be tied to a single strain of BVDV. Surprisingly, the amount of change with increasing time and distance was relatively small, ranging from 0.04 to 0.3% at the nucleotide level. It was obvious that with a longer time between isolations, the greater the number of genomic changes. The length of time between isolation of the original IAF-103 virus and last virus isolated (37621) was estimated at greater than one year. An unexpected finding was that greater number of nucleotide and amino acid changes occurred in the nonstructural proteins rather than the structural proteins. The number of cattle infected by each virus is unknown but it seems logical to assume that at some point these viruses would have infected animals with prior vaccination or previously infected with other strains of BVDV, putting immune pressure on the immunodominant residues of the SA viruses. Conventional wisdom states that it is this immune pressure that drives genetic change, especially in the structural proteins. From data presented here, there appears to have been little pressure to change the E2 protein to evade an immune response. Earlier work by Tang and Zhang (2007) showed that positive selection of mutations to avoid antibody recognition was not an important factor in the evolution of BVDV. Antigenic comparisons with a 27 monoclonal antibody panel showed that there were no antigenic differences in these viruses.

In addition to acute infections that can move rapidly through and between cattle herds, noncytopathic strains of BVDV can establish life-long persistent infections in utero and be spread by the persistently infected animal for life. There has long been debate whether the acute or the persistent infection has the higher nucleotide substitution rate, resulting in new BVDV strains with altered characteristics. The persistent viruses examined in this study showed a more rapid rate of change then that observed in the SA viruses. In a single in vivo passage, these viruses displayed nucleotide and amino acid substitution rates equal to or greater than that observed in the most divergent SA viruses. Most of these changes occurred in the structural proteins, particularly the E2 protein, suggesting that establishment of the persistent infection may play an important role in generating antigenic diversity, even in the absence of an immune response (Borrego et al., 1993; Domingo et al., 1993; Sevilla et al., 1996). Two antibodies of the E2 monoclonal antibody panel showing altered antigen recognition patterns in the two type 1b progeny viruses supports this. Additionally, some amino acid changes in the E2 protein were common to all progeny viruses within genotype indicating that there may be some selection mechanism, perhaps genetics of the host, that drives the selection of specific changes in the E2 protein.

Prior work indicated that both acute and persistent infections result in changes in nucleotide sequence but it was suggested that acute infections were more likely the driver of sequence diversity, particularly in the E2 protein, because of immune pressure on the virus (Bolin et al., 1991). Thus, most of these studies focused on the nucleotide sequence of the immunodominant E2 protein, assuming that the E2 protein was the main site of sequence diversity, and ignored other regions of the genome. (Stokstad et al., 2004) followed changes that occurred in the E2 protein between the biologically cloned virus stock used to infect pregnant dams and the 15 viruses isolated from the resulting PI animals. They noted some changes in a 744 base variable region of the E2 protein, ranging from 0 to 6 nucleotides. This is in agreement with the rate of nucleotide substitutions presented here. However, earlier studies looking at small regions of the genome missed the amount of genetic change that occurred in the establishment of the PI state. Sequencing the entire ORF demonstrated that nucleotide substitutions occurred throughout the genome, apparently randomly, with a smaller percentage resulting in amino acid changes. The amino acid changes were probably not random and were found in regions with little or no structural or functional constraints. Additionally, a number of changes occurred in the genome of the PI progeny viruses with some resulting in detectible antigenic differences in the type 1b progeny viruses. It is unknown at this point if the sequence of the progeny PI virus was represented in the quasispecies found in the progenitor PI calf or if these changes were introduced into the virus during the establishment of the persistent infection in the progeny PI calf. This will be the focus of future experiments.

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